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Adenovirus Origin of DNA Replication: Sequence Requirements for Replication In Vitro

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The initiation of adenovirus DNA takes place at the termini of the viral genome and requires the presence of specific nucleotide sequence elements. To define the sequence organization of the viral origin, we tested a large number of deletion, insertion, and base substitution mutants for their ability to support initiation and replication in vitro. The data demonstrate that the origin consists of at least three functionally distinct domains, A, B, and C. Domain A (nucleotides 1 to 18) contains the minimal sequence sufficient for origin function. Domains B (nucleotides 19 to 40) and C (nucleotides 41 to 51) contain accessory sequences that significantly increase the activity of the minimal origin. The presence of domain B increases the efficiency of initiation by more than 10-fold in vitro, and the presence of domains B and C increases the efficiency of initiation by more than 30-fold. Mutations that alter the distance between the minimal origin and the accessory domains by one or two base pairs dramatically decrease initiation efficiency. This critical spacing requirement suggests that there are specific interactions between the factors that recognize the two regions.

The replication of the adenovirus genome has been studied extensively as a means of probing the mechanisms of DNA synthesis in animal cells (reviewed in reference 24). The viral genome is a linear, duplex DNA molecule of about 35,000 base pairs (bp). The extreme termini of the genome have identical nucleotide sequences and contain the viral origins of DNA replication (2, 10, 13, 19, 26, 46, 47; P. J. Rosenfeld, R. J. Wides, M. D. Challberg, and T. J. Kelly, Jr., *in Cancer Cells-DNA Tumor Viruses, vol. 4*, in press). Studies of the replication process in vivo have demonstrated that initiation of DNA synthesis takes place at the two ends of the viral genome with about the same frequency (14, 27). After each initiation event, a daughter strand is synthesized in the 5′-to-3′ direction, displacing one of the parental strands. Upon completion of the first daughter strand, the displaced parental strand serves as template for the synthesis of a second daughter strand.

The development of a cell-free replication system (7) and subsequent in vitro studies in several laboratories (6, 9, 12, 15, 30, 35, 45) have provided considerable insight into the mechanism of initiation of adenovirus DNA replication. The fundamental initiation reaction is the formation of a covalent linkage between dCMP, the first residue in the new daughter strand, and a virus-encoded primer protein, designated the adenovirus preterminal protein (pTP). The formation of the dCMP-pTP initiation complex is dependent on the presence of adenovirus DNA and requires the participation of both viral and cellular replication proteins (see below). Once the complex has formed, elongation of the new daughter strand proceeds by the addition of nucleotides at the free 3′ OH group of the dCMP residue that is covalently bound to the pTP.

Fractionation of the cell-free system has led to the identification and purification of several proteins required for viral DNA replication. Three of the required proteins are encoded by the adenovirus genome. Of these, the 80-kilodalton (kDa) preterminal protein (pTP) (6, 43) and the 140-kDa adenovirus polymerase (15, 16, 35, 44) have been shown to be involved in the initiation reaction (15, 35, 43). The third virus-encoded replication protein, the 72-kDa single-stranded DNA-binding protein (48), is dispensable for initiation, but is required, together with the adenovirus polymerase, for elongation of nascent DNA chains (9, 15, 35, 41, 44, 45). Initiation of adenovirus DNA replication has also been shown to be dependent on a cell-encoded protein, nuclear factor I (NF-I) (32, 33, 38, 40, 41). NF-I is a sequence-specific DNA-binding protein that interacts with a site located near the termini of the viral genome. While the interaction of NF-I with its recognition site has been extensively documented (13, 18, 23, 28, 33, 38, 41; Rosenfeld et al., in press), the precise biochemical role of NF-I in the initiation reaction, as well as its normal function in the uninfected cells, remain unknown.

Previous studies have demonstrated that specific nucleotide sequence domains at the terminus of the adenovirus genome are essential for efficient initiation of DNA replication. These sequence domains have been partially defined by studying the replication of plasmid templates containing cloned DNA segments derived from the termini of the viral genome (10, 13, 18, 19, 26, 28, 38, 46, 47). In vitro studies have demonstrated that the adenovirus origin of replication is wholly contained within the terminal 67 nucleotides of the genome. It has also been shown that the first 18 nucleotides of the genome are sufficient to support a limited degree of initiation but that the presence of the region between nucleotides 19 and 67 greatly enhances the efficiency of initiation (13, 19, 38). One important element that lies within the latter region is the recognition site for NF-I (13, 18, 23, 28, 33, 38,

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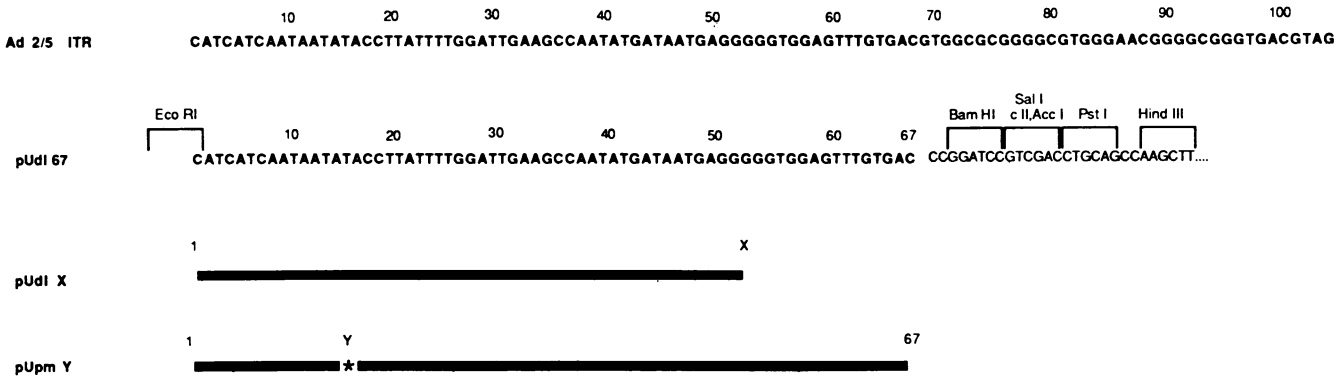

FIG. 1. Sequence of the adenovirus terminus and nomenclature of mutants. The terminal sequence of Ad2 and Ad5 is shown at the top of the figure. Directly below is the terminal sequence of the plasmid pU*dl*67 linearized by cleavage with *Eco*RI. Boldface letters indicate Ad2 and Ad5 sequences. Lightface letters indicate pUC9 polylinker sequences. Like pU*dl*67, all the mutant plasmids used in this study contain single segments of the adenovirus terminus inserted into the polylinker site of pUC9. Mutants are named according to the following conventions. The designation pU*dlX* refers to a deletion mutation (*dl*) that contains nucleotides 1 to *X* to the adenovirus terminal sequence. The designation pUpm*Y* refers to a point mutation (pm) with a base substitution at nucleotide *Y* of the adenovirus terminal sequence. Most of the point mutations were generated in plasmids containing nucleotides 1 to 67 of the viral terminus. However, three point mutations (pUpm4, pUpm17, and pUpm18) were in plasmids containing nucleotides 1 to 73. ITR, Inverted terminal repetition.

41; Rosenfeld et al., in press). The general picture of the organization and extent of the adenovirus origin region that has emerged from the in vitro studies is consistent with results from recent in vivo studies (22, 50).

In this paper we report the results of a detailed genetic study of the adenovirus origin of DNA replication. Analysis of the ability of a large number of deletion and point mutants to support initiation and replication in vitro indicates that the sequence organization of the viral origin is more complex than previously suspected. The origin contains at least three functionally distinct domains: the minimal origin and two adjacent accessory domains, both of which are required for optimal levels of initiation and replication. Our results also indicate that the distance between the minimal origin and the two accessory domains is critically important for efficient initiation.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA polymerase, *Micrococcus luteus* DNA polymerase, T4 DNA ligase, S1 nuclease, exonuclease III, and calf intestinal alkaline phosphatase were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England BioLabs, Inc. (Beverly, Mass.), P-L Biochemicals, Inc. (Milwaukee, Wis.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or International Biotechnologies, Inc. "Slow" *Bal* 31 exonuclease was purchased from International Biotechnologies. Oligonucleotides were purchased from Bethesda Research Laboratories or were synthesized by Clark Riley and Ching-Ming Tseng of the Howard Hughes Laboratory of Genetics, Baltimore, Md. The radioactive nucleotides [α-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from New England Nuclear Corp. (Boston, Mass.) at 3,000 Ci/mmol.

Plasmid constructions. Plasmid constructions were done by standard methods (31) except when indicated. The detailed procedures have been described elsewhere (R. J. Wides, Ph.D. thesis, The Johns Hopkins University, Baltimore, Md., 1986). A summary of the nomenclature for plasmids carrying adenovirus origin mutations is presented in Fig. 1. Each plasmid carried a segment of DNA derived from the extreme terminus of the adenovirus type 5 (Ad5) genome. In every case the viral DNA segment was inserted into the polylinker site of pUC9 to ensure a constant genetic background. Deletion mutations are named according to the location of the internal deletion endpoint (i.e., pU*dl*67 contains the adenovirus sequence from nucleotide 1 to nucleotide 67). Point mutations are named according to the position of the base substitution (i.e., pUpm19 contains a base substitution at nucleotide 19).

The deletion mutants pU*dl*7, -12, -18, -31, -36, and -67 are subclones of *dl*7, -12, -18, -31, -36, and -67, respectively, which have been described previously (38). In each case the starting plasmid was digested with *Eco*RI and *Bam*HI, and the fragment containing the adenovirus terminal sequence was inserted between the *Eco*RI and *Bam*HI sites of pUC9. A second series of deletion mutants was constructed starting with the plasmid pU*dl*67. The plasmid DNA was cleaved with *Bam*HI, and the resulting unit-length linear molecules were digested with the slow isozyme of *Bal* 31 exonuclease. The digestion products were incubated with T4 DNA polymerase in the presence of the four deoxyribonucleoside triphosphates to ensure that the deletion endpoints had blunt ends. After the addition of *Bam*HI linkers, the DNA was digested with *Bam*HI and *Eco*RI, and the adenovirus terminal fragment was cloned between the *Bam*HI and *Eco*RI sites of pUC9. This procedure resulted in the generation of mutants pU*dl*42, pU*dl*45, pU*dl*51, pU*dl*52, pU*dl*54, pU*dl*55, and pU*dl*56.

The deletion mutants pU*dl*40 and pU*dl*41 were constructed from the *Mnl*I (blunt end) to *Eco*RI adenovirus terminal fragment (adenovirus sequences 1 to 40). The vector pUC9 was digested with *Sal*I and incubated with T4 DNA polymerase in the presence of the four deoxynucleoside triphosphates to convert the *Sal*I end to a blunt end. After digestion with *Eco*RI, the large linear vector fragment was joined to the 40-bp adenovirus terminal fragment to generate pU*dl*40. Plasmid pU*dl*41 was also recovered, presumably because of incomplete blunt-end conversion at the *Sal*I site.

Nearly all the point mutants were identical to pU*dl*67 except for the presence of a single base substitution. The point mutants pUpm19 and pUpm20 were constructed by oligonucleotide-directed mutagenesis by a modification of the method of Zoller and Smith (51, 52). Point mutants pUpm26/57, pUpm27/65, pUpm31, pUpm42, pUpm52,

pUpm54, and pUpm57 were prepared by the bisulfite method of Shortle and Nathans (42). Point mutants pUpm21C, pUpm21T, pUpm24, pUpm25, pUpm28, pUpm29, pUpm30, pUpm32, pUpm33, pUpm34C, pUpm34T, pUpm35, pUpm36, pUpm37, pUpm38, and pUpm40 were prepared by the synthetic oligonucleotide mutagenesis procedure of Norris et al. (34). Point mutants pUpm4, pUpm17, and pUpm18 were derived from pMDC10-pm4, pMDC10-pm17, and pMDC10-pm18, respectively, which have been described previously (38). The starting plasmids were cleaved with *Eco*RI and *Hin*PI, and the fragment containing the terminal 72 bp of the adenovirus genome was ligated to the *Eco*RI-to-*Acc*I fragment of pUC9.

The nucleotide sequence of each deletion and point mutant was determined by the dideoxynucleotide method. Plasmid templates were prepared for sequence analysis by either the exonuclease III method (20) or the alkali denaturation method (11). Plasmids were propagated in *Escherichia coli* JM83 (49) and DHI. Ad5 DNA was prepared as described by Challberg and Kelly (7).

Assay of DNA replication in vitro. Nuclear extracts were prepared from Ad5-infected cells as described by Challberg and Kelly (7) and by Ostrove et al. (35). Unwashed nuclei were extracted with 0.2 M NaCl, and the resulting extract was frozen at −80°C. The standard in vitro replication reaction mixture (25 μl) contained 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 2% dimethyl sulfoxide, 2 mM ATP, 25 μM [α-^{32}P]dCTP at a specific activity of 18 Ci/mmol, 25 μM each dATP, dGTP, and dTTP, 20 μg of plasmid DNA per ml, and 6 μl of unfractionated nuclear extract. Before the assay the mutant plasmid templates were digested with *Eco*RI and *Ava*II, extracted with phenol-chloroform, collected by precipitation with ethanol, and suspended in 10 mM Tris hydrochloride–1 mM EDTA (pH 8.0). DNA concentrations were measured by the diphenylamine method (5) to ensure that interference owing to contaminating RNA did not affect the concentration determination. Replication reaction mixtures were incubated at 37°C for 80 min and were stopped by the addition of sodium dodecyl sulfate (SDS) to 1% and EDTA to 25 mM. The reaction products were collected by ethanol precipitation, dissolved in 1% SDS–10 mM EDTA (pH 8.0), and fractionated by electrophoresis through a 1.4% agarose gel in a buffer containing 0.1% SDS. After electrophoresis, the gel was dried, and the radioactivity was located by autoradiography. Radioactive bands were excised from the gel and quantitated by direct scintillation counting in nonaqueous fluor. The background was determined by excising gel slices of equivalent size from several different positions in the gel.

Spacing mutations. The progenitor of all spacing mutations in the *dl*46 series, pU*dl*46I26, was constructed by inserting an NF-I site created with synthetic oligonucleotides into pU*dl*18. A pair of complementary 28-nucleotide oligomers containing adenovirus terminal nucleotides 19 through 46 flanked by a *Pst*I site on the left and a *Bgl*II-*Pst*I site on the right were used for this construction. The oligonucleotides were hybridized and cloned into the *Pst*I sites of pUC4KSAC (4), yielding the plasmid pUC4BS. Plasmid pUC4BS was digested with *Bam*HI, and the fragment containing the NF-I site was isolated and inserted into the *Bam*HI site of pU*dl*18, yielding pU*dl*18BS-3. Finally, pU*dl*18BS-3 was digested with *Hin*dIII and *Bgl*II, treated with *M. luteus* DNA polymerase to convert the restriction sites to blunt ends, and circularized in the presence of *Bgl*II linkers. The resulting plasmid, pU*dl*46I26, contained the adenovirus minimal origin (adenovirus base pairs 1 through 18) and the NF-I-binding site (adenovirus base pairs 19 through 46) in the same orientation, separated by 26 nucleotides (see Fig. 8A).

Plasmid pU*dl*46I26 contains unique *Bam*HI and *Pst*I restriction sites between the minimal origin and the NF-I-binding site. By cleaving at one or more of these sites and generating blunt ends, we constructed a number of mutants with altered spacings between the minimal origin and the NF-I site (see Fig. 8A). Blunt ends were created by treating with T4 DNA polymerase to extend the recessed 3′ strand in the *Bam*HI site or to remove the 4-bp 3′ single-stranded overhang in the *Pst*I site. Plasmid pU*dl*46I30 (see Fig. 8A) was constructed by linearizing pU*dl*46I26 with *Bam*HI and treating with T4 DNA polymerase. Plasmid pU*dl*46I22 was constructed by linearizing pU*dl*46I26 with *Pst*I and treating with T4 polymerase. Plasmid pU*dl*46I8 was constructed by digesting pU*dl*46I26 DNA with both *Pst*I and *Bam*HI followed by incubation with T4 DNA polymerase. In addition to the expected product, pU*dl*46I8, the plasmid pU*dl*46I9, which had a *Bam*HI restriction site in the spacer DNA, was also recovered. Plasmid pU*dl*46I13 was constructed by linearizing pU*dl*46I9 with *Bam*HI and treating with T4 DNA polymerase.

Blunt ends were also created by using S1 nuclease to remove the 5′ or 3′ single-stranded overhangs in restriction sites. The plasmid pU*dl*46I4 was constructed by digesting 1 μg of pU*dl*46I26 DNA with *Bam*HI and *Pst*I followed by incubation with 1 U of S1 nuclease for 5 min at 25°C in a 10-μl reaction mixture. The plasmid pU*dl*46I5 was constructed by digesting 1 μg of pU*dl*46I9 DNA with *Bam*HI followed by incubation with 1 U of S1 nuclease for 5 min at 25°C in a 10-μl reaction mixture. When larger amounts of S1 nuclease (10 U) were used under otherwise identical conditions, nucleotides were removed from the ends of duplex DNA at a low rate. Using this approach we obtained a collection of spacing mutations that included pU*dl*46I1, pU*dl*46I2, pU*dl*46I3, pU*dl*46I4, pU*dl*46I5, Δ4, and Δ7. The plasmid pU*dl*46I0 was not found among these clones.

A more direct method was used for constructing pU*dl*46. A restriction fragment containing wild-type adenovirus DNA was used to form a heteroduplex with an M13 clone containing adenovirus sequences from pU*dl*46I1. The M13 clone, M13mp9*dl*46I1, contained the *Eco*RI to *Bgl*II adenovirus fragment of pU*dl*46I1 inserted between the *Eco*RI and *Bam*HI sites of M13mp9. The restriction fragment contained nucleotides 1 to 40 of the adenovirus genome plus 140 nucleotides of pUC9 sequence adjacent to adenovirus nucleotide 1. The duplex fragment was denatured and hybridized to the M13 clone. The annealed strand was extended by incubation with the Klenow fragment of DNA polymerase I and the four deoxynucleoside triphosphates. The synthetic product was digested with *Eco*RI and *Hin*dIII, and the resulting heteroduplex adenovirus fragment was inserted between the *Eco*RI and *Hin*dIII sites of pUC9. The resulting construct was designated pU*dl*46HindIII. The plasmid pU*dl*46 was constructed by excising a segment containing the first 46 bp of pU*dl*46HindIII by cleavage with *Eco*RI and *Sau*3AI and joining it to the large *Eco*RI-to-*Bgl*II fragment of pU*dl*46I22.

The plasmids pU*dl*67I1, pU*dl*67I2, pU*dl*67I3, pU*dl*67I4, pU*dl*67I5, Δ4, and Δ7 were derived from the corresponding spacing mutations in the *dl*46 series. In each case, the pU*dl*46 spacing mutant was digested with *Sau*3AI, and the fragment containing the origin was isolated. The purified fragment was denatured and hybridized to an M13 clone

containing the terminal 67 nucleotides of adenovirus (M13mp9*dl*67). The 3′ end of the annealed fragment at nucleotide 46 was extended to beyond nucleotide 67 by incubation with the Klenow fragment and the four deoxynucleoside triphosphates. The products of this reaction were digested with *Eco*RI and *Bam*HI, and the resulting heteroduplex fragment was inserted between the *Eco*RI and *Bam*HI sites of pUC9.

Plasmids pU*dl*67Δ1 and pU*dl*67Δ2 were constructed by a modification of the method of Zoller and Smith (51, 52) with 18-nucleotide synthetic oligomers and the M13 clone M13mp9*dl*67. Plasmids pU*dl*67I10 and -I11 were constructed by a modification of the method of Zoller and Smith (51, 52) with 30-nucleotide synthetic oligomers and the M13 clone M13mp9*dl*67I5. The mutations pU*dl*67I9, -I14, and -I15 were constructed from pU*dl*67I10 and -I11 by utilizing unique *Sac*II and *Bam*HI sites located between the minimal origin and the NF-I site. Using procedures similar to those used to generate pU*dl*46 spacing mutations, the unique restriction sites were altered to remove or insert spacer DNA.

Assay of pTP-dCMP complex formation in vitro. Standard reaction mixtures (25 μl) contained 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 3 mM ATP, 0.5 μM [α-^{32}P]dCTP (3,000 Ci/mmol; 1 Ci = 3.7×10^{10} Bq), 40 μg of aphidicolin per ml, 20 μg of plasmid DNA per ml (digested with *Ava*II and *Eco*RI), and 6 μl of unfractionated nuclear extract from Ad5-infected cells. After incubation for 90 min at 30°C, the reaction mixtures were supplemented with $CaCl_2$ to 10 mM and incubated for 45 min at 37°C with 5 U of micrococcal nuclease. The reaction products (pTP-dCMP complexes) were immunoprecipitated with serum directed against the adenovirus terminal protein (gift of M. Green [17]). Samples were brought to 20 mM EDTA, and then 175 μl of immunoprecipitation buffer was added (0.35% Triton X-100, 0.35% Nonidet P-40, 13 mM EDTA, 1.3 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline, pH 7.2). After the addition of 10 μl of rabbit antiserum directed against the adenovirus terminal protein, the samples were incubated for 2 h at room temperature. Protein A-Sepharose beads equilibrated with immunoprecipitation buffer (25 μl) were added, and the incubation was continued with rocking for 2 h at 4°C. After incubation, the beads were washed two times with a buffer containing 50 mM Tris hydrochloride (pH 7.5), 5 mM EDTA, 0.5% Nonidet P-40, 200 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. The immunoprecipitated proteins were eluted from the beads by incubation in 25 μl of sample buffer (50 mM Tris hydrochloride [pH 6.8], 10% glycerol, 0.3 M 2-mercaptoethanol, 2% SDS, 0.005% phenol red) for 5 min at 100°C. Samples were then electrophoresed on 10% SDS–polyacrylamide gels (25). After electrophoresis, the gels were dried, and the radioactivity was located by autoradiography.

RESULTS

Assay for replication of DNA fragments containing adenovirus origins of replication. To analyze the nucleotide sequence requirements for adenovirus DNA replication, we constructed a large number of plasmids with deletions or point mutations in the viral terminal sequence and assayed their ability to support DNA replication in vitro. The initial starting point for these constructions was the plasmid pXD7, which contains the left terminal *Xba*I restriction fragment from Ad2 cloned into the *Eco*RI site of pBR322 (36). When pXD7 (or any of the derivatives in the present study) is digested with *Eco*RI the product of cleavage is a linear molecule with the adenovirus origin at one terminus. The nucleotide sequence at this terminus is identical with the terminal sequence of Ad2 (or Ad5), except for the presence of an additional four nucleotides at the 5′ end of one strand. It has been shown previously that restriction fragments with this structure can serve as effective templates for DNA replication in vitro (10, 19, 26, 38, 46, 47). The products of replication of such molecules are easily distinguished from the input DNA fragments because they have a reduced electrophoretic mobility in SDS-agarose gels. This is a consequence of the presence of the covalently bound adenovirus terminal protein that serves as the primer for DNA replication. In the studies reported here the relative incorporation of radioactive precursor into DNA fragments linked to the pTP was taken as a direct measure of the relative efficiency of DNA replication (38). Incorporation of radioactive precursor into fragments unlinked to the pTP was assumed to represent a repairlike reaction.

Replication of deletion mutants. Plasmids containing deletion mutations in the cloned adenovirus terminal sequences were digested with *Eco*RI and *Ava*II before their use as templates for in vitro DNA replication. The cleavage products included a fragment about 1,450 bp in length that contained the adenovirus origin of replication positioned at one end. The exact size of this fragment was dependent on the size of the deletion mutation. The two other cleavage products (1,023 and 222 bp) contained only pUC9 DNA sequences. As expected, all three input DNA fragments incorporated some radioactivity as a result of repair synthesis. However, when the largest fragment contained a functional adenovirus origin of replication, a new radioactive species with a slightly lower mobility was observed (Fig. 2). This species comigrated with the largest input DNA fragment after digestion with pronase, indicating that it contained the covalently bound pTP (data not shown).

Comparison of the replication activities of the various deletions revealed the existence of four distinct classes of mutants, each with a characteristic replication efficiency (Fig. 2). All mutant templates that contained at least 51 bp of the adenovirus terminus replicated to approximately the same extent in vitro. These templates included pU*dl*51, pU*dl*52, pU*dl*54, pU*dl*55, pU*dl*56, and pU*dl*67. Similar replication efficiencies were observed for templates that contained over 350 bp of the left (pMDC10) or right (pRER) terminus of the adenovirus genome. We conclude that the wild-type adenovirus origin of DNA replication is entirely contained within the terminal 51 nucleotides. Templates that contained between 40 and 46 bp of the viral terminus (pU*dl*40, pU*dl*41, pU*dl*42, pU*dl*45, and pU*dl*46) replicated with significantly lower efficiency than the longer templates. Thus, the internal boundary of the origin lies between nucleotide 46 and nucleotide 51. Templates that contained between 18 and 36 bp of the viral terminus (pU*dl*18, pU*dl*31, and pU*dl*36) replicated with very low efficiency, but small amounts of pronase-sensitive replication products could be observed upon long exposure of the autoradiogram (Fig. 2B). Templates that contained less than 18 bp of the viral terminus (pUC9, pU*dl*7, and pU*dl*12 [data not shown]) did not support any detectable DNA replication in vitro.

The replication activities of the deletion mutants were quantified at four different input DNA concentrations: 10, 20, 30, and 40 μg/ml. At each concentration the extent of incorporation of radioactive precursor into pronase-sensitive replication products was determined by direct scintillation counting after excision of the appropriate bands from the agarose gel. The replication activities of the mutants in-

creased with increasing DNA concentration (Fig. 3), but the activity of any given mutant relative to the others was essentially independent of template concentration. The data indicate that templates containing between 40 and 46 bp of the adenovirus terminus supported about 30 to 50% of the DNA synthesis observed with templates containing the complete adenovirus origin of replication (i.e., more than 50 bp of the viral terminus). Templates containing between 18 and 36 bp of the viral terminus supported less than 10% of the DNA synthesis seen with the complete origin.

Initiation of adenovirus DNA replication on templates containing deletions in the origin. To verify that the deletion mutations specifically affected the initiation step of adenovirus DNA replication, we determined the ability of the mutant templates to support the formation of pTP-dCMP complexes in vitro. For this purpose the plasmid DNA molecules were cleaved with *Eco*RI and *Ava*II and then incubated with nuclear extract from Ad5-infected cells in the presence of

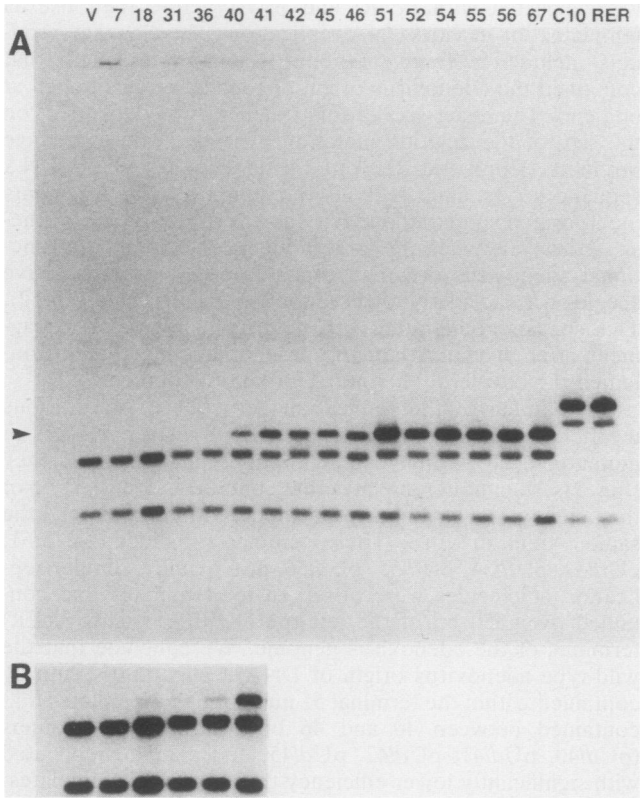

FIG. 2. Replication of deletion mutants. (A) Plasmids were digested with *Eco*RI and *Ava*II, and the resulting fragments were incubated with nuclear extract from Ad5-infected cells in a standard in vitro DNA replication reaction mixture. The radioactive DNA product was isolated and analyzed by agarose gel electrophoresis in a buffer containing 0.1% SDS. The largest restriction fragment (approximately 1,450 bp) contains the adenovirus terminus positioned at one end. The arrowhead marks the position of the major replication product, which consists of a covalent complex between the largest fragment and the adenovirus preterminal protein (38). The numbers above each lane refer to the deletion endpoint of the corresponding DNA template. Lanes: V, vector pUC9 DNA as the template; RER; plasmid as the template (pRER contains 358 bp of the right terminus of the Ad5 genome); C10, plasmid pMDC10 as the template (pMDC10 contains 358 bp of the left terminus of the Ad5 genome). (B) A fivefold-longer autoradiographic exposure of lanes V through 40.

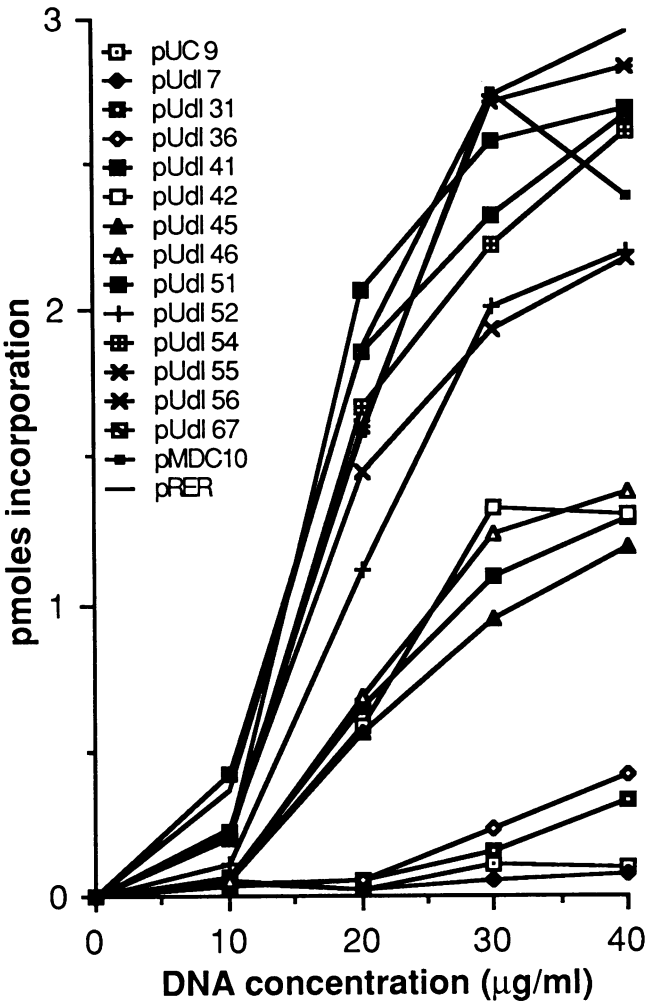

FIG. 3. Replication of deletion mutants as a function of DNA concentration. Replication reactions were done under standard conditions, except for the concentrations of the DNA templates (10, 20, 30, and 40 μg/ml). The replication products were separated on agarose gels in the presence of 0.1% SDS. The radioactive fragment containing the adenovirus origin linked to the pTP was excised from the gel, and the extent of incorporation was quantitated by scintillation counting. One picomole of incorporation represents 4,500 cpm.

[α-^{32}P]dCTP (6, 9, 30, 37, 45). The reaction conditions were essentially identical to those used for the replication reactions described above except for the absence of deoxynucleoside triphosphates other than dCTP. The radioactive pTP-dCTP complexes formed in the reaction were recovered by immunoprecipitation with antibody to the adenovirus terminal protein and analyzed by SDS-polyacrylamide gel electrophoresis.

The results of the initiation assays were in complete agreement with those obtained with the replication assays (Fig. 4). In particular, the maximal level of initiation was observed with templates that contained at least 51 bp of the adenovirus terminus (lanes 51 through C10). Templates containing 40 to 46 nucleotides of the terminus supported initiation at significantly lower efficiencies (lanes 40 through 46). Templates containing from 18 to 36 nucleotides of the terminus supported very low, but detectable levels of initiation (inset B, lanes 18 through 36), while templates contain-

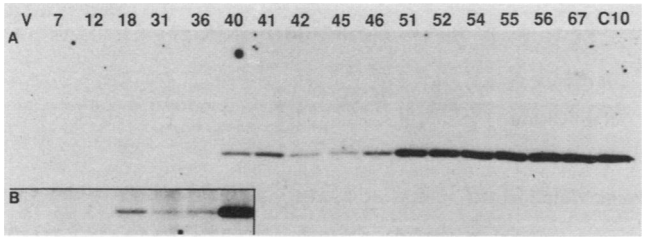

FIG. 4. Initiation of replication with deletion mutants. Each mutant plasmid was cleaved with *Eco*RI and *Ava*II to generate a linear DNA fragment with the adenovirus terminus at one end. The resulting templates were tested for their ability to support synthesis of 80-kDa pTP-dCMP complexes in vitro. Standard reaction mixtures contained nuclear extract from Ad5-infected cells and [α-^{32}P]dCTP as the only deoxynucleoside triphosphate. Radioactive pTP-dCMP complexes were collected by immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis. (A) Lanes are labeled with the deletion endpoints as in Fig. 2. The major band on the autoradiograph has the mobility expected of an 80-kDa protein. (B) A sevenfold-longer autoradiographic exposure of lanes V through 40.

ing less than 18 bp of the terminus completely failed to support initiation (lanes V through 12).

The data on the initiation and replication of deletion mutants support the model for the sequence organization of the adenovirus origin that is shown in Fig. 5. In this model the origin consists of three discrete sequence domains, labeled A, B, and C. Domain A constitutes the minimal sequence that is sufficient to support initiation of adenovirus DNA replication (10, 19, 26, 38, 46, 47), and domains B and C function to increase the efficiency of initiation. Domain B is known to contain a high-affinity binding site for the cellular protein NF-I (19, 32, 38). As shown in the accompanying paper (41), domain C contains the recognition site of another cellular DNA-binding protein.

Initiation and replication of DNA fragments containing point mutations in adenovirus origin. To confirm and extend the data obtained with deletion mutants, we examined the effects of base substitution mutations in each of the three sequence domains of the adenovirus origin of replication. The mutations were generated by several different in vitro mutagenesis procedures; however, the final plasmid constructs all contained 67 to 73 bp of the adenovirus terminus cloned into the polylinker site of pUC9. After cleavage with *Eco*RI and *Ava*II the mutant plasmids were assayed for their ability to support initiation (Fig. 6) or complete replication (summarized in Fig. 7). The results of the two different assays were in good agreement and were also consistent with the data derived from analysis of the deletion mutants.

Templates with point mutations at nucleotides 17 or 18 in domain A (pUpm17 and pUpm18) greatly reduced the efficiency of initiation and replication. Both mutations affect a sequence within the minimal origin that is highly conserved among all adenovirus serotypes. Three mutations in domain B (pUpm26, pUpm35, and pUpm36) reduced the extent of initiation and replication to about the level observed with the minimal origin alone. These mutations affect nucleotides that reside within the consensus recognition site for NF-I (TTG G^C_ANNNNNGCCAA) and are also highly conserved among the various adenovirus serotypes (18, 23). A number of other mutations within the NF-I recognition site (e.g., pUpm24, pUpm25, and pUpm37) had small, but reproducible, effects on replication efficiency. As expected, mutations in the 5-bp spacer between the two symmetrical halves of the NF-I recognition site had no detectable effect on initiation or replication in vitro. Finally, two mutations in domain B (pUpm20 and pUpm21) which affected nucleotides outside the NF-I recognition site reduced replication efficiency to about 50% of the control value. All other mutations in domain B that were tested had no effect on initiation or replication (Fig. 6 and 7). Two base substitutions in domain C (pUpm40 and pUpm42) reduced the extent of initiation and replication by 50 to 70% in good agreement with the data obtained with deletion mutants containing lesions in domain C.

Effect of altered spacing between minimal origin of replication and accessory sequence elements. The data presented in this paper, together with previous studies (19, 38, 47), indicate that the first 18 bp of the adenovirus terminus (domain A) are sufficient to support a limited degree of

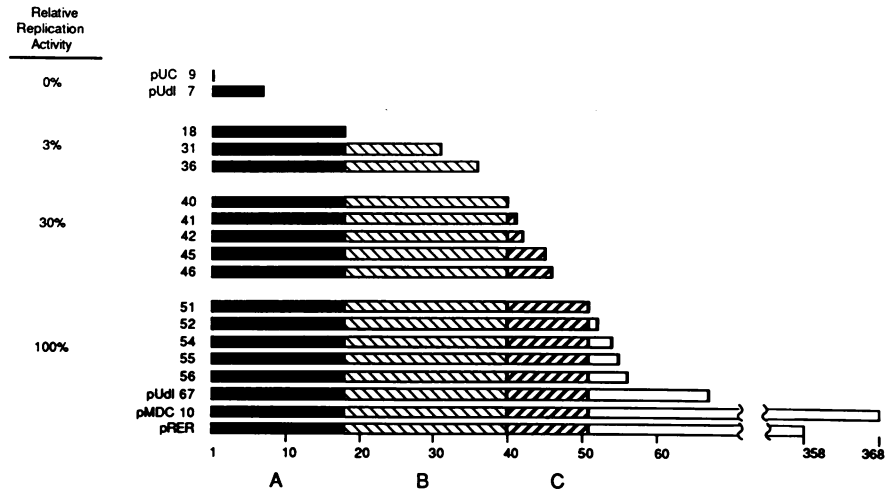

FIG. 5. Schematic representation of the tripartite adenovirus origin of replication. The deletion mutants analyzed in this study are classified into four distinct groups on the basis of their ability to support adenovirus DNA replication in vitro. The relative replication activities of each group (at an input DNA concentration of 20 μg/ml) are shown to the left. The values on the abscissa represent the distance from the terminus of the adenovirus genome in base pairs. The initiation and replication data of Fig. 2 to 4 are accomodated by a model in which the origin is composed of three functionally distinct domains, A (solid bar), B (light diagonals), and C (bold diagonals).

initiation but that the adjacent accessory domains B and C are required for optimal initiation efficiency. It was of interest to determine whether the distance between the minimal origin and the accessory domains is critical for efficient origin function. As an approach to this question we constructed mutants containing insertions of various lengths between nucleotides 18 and 19. The site of insertion was chosen to minimize the disruption of domains A and B. The data presented above indicate that pU*dl*18 replicates as efficiently as pU*dl*36, suggesting that nucleotides outside of the first 18 are not important for minimal origin function. Moreover, we have demonstrated that the DNA segment from nucleotide 19 to nucleotide 46 binds to NF-I with the same affinity as the complete origin of replication (data not shown).

Two series of insertion mutants were constructed (Fig. 8). One series contained the minimal origin and domain B as the only functional accessory domain (*dl*46 series), and the other series contained the minimal origin and both accessory domains (*dl*67 series). Both series of mutants were assayed for their ability to support initiation of replication under standard conditions in vitro (Fig. 9). For the *dl*46 series, insertion of one to four nucleotides between domains A and B greatly reduced the efficiency of initiation (Fig. 9A). Similar results were obtained with the *dl*67 series, although, as expected, the presence of domain C significantly increased the initiation efficiencies of the wild-type template as well as some of the mutants (Fig. 9B). The insertion of a single base pair (*dl*67I1) reduced initiation to about half the level observed with the wild-type template (*dl*67), while insertion of one to three additional nucleotides (*dl*67I2 to *dl*67I4) reduced initiation to very low levels. We conclude from these results that the precise spacing of domains A and B is critically important for optimal origin function. Examination of the initiation efficiencies of mutants with small deletions in the neighborhood of nucleotide 19 provided additional support for this conclusion (Fig. 8 and 9). Deletion of nucleotide 19 (*dl*67Δ1) reduced initiation to about 30% of the wild-type level, and deletion of nucleotides 19 and 20 (*dl*67Δ2) completely abolished initiation.

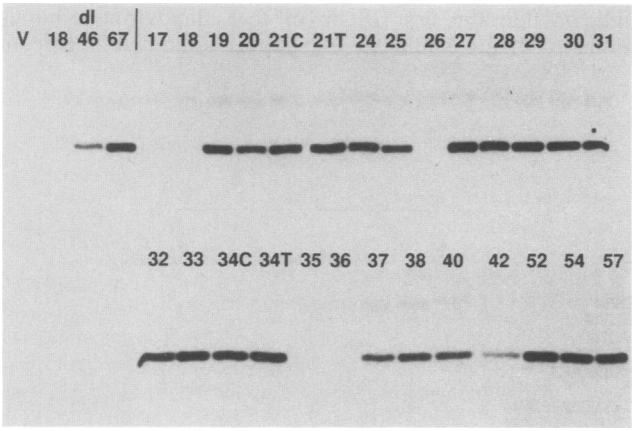

FIG. 6. Initiation of replication with point mutants. Templates derived from plasmids containing point mutations in the cloned viral terminal sequence were assayed for their ability to support synthesis of 80-kDa pTP-dCMP complexes in vitro as described in the legend to Fig. 4. For purposes of comparison, lanes V, *dl*18, *dl*46, and *dl*67 show the results of identical initiation assays with representatives of each of the four classes of deletion mutations (pUC9, pU*dl*18, pU*dl*46, and pU*dl*67, respectively). The numbers above the remaining lanes refer to the position within the viral terminal sequence of the base substitution mutation (e.g., the template used for lane 19 was pUpm19, which has a single base pair substitution at adenovirus nucleotide 19).

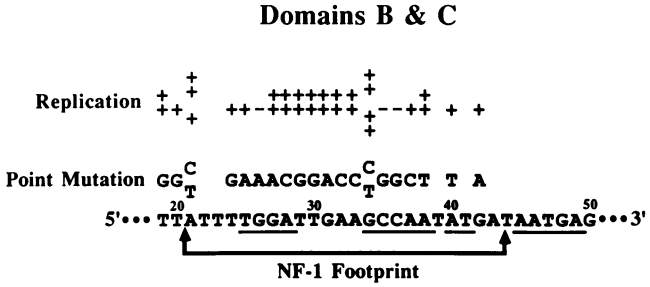

FIG. 7. Replication of point mutants. Plasmids with point mutations in the cloned viral terminal sequence were assayed for replication over a range of DNA concentrations as described in the legend to Fig. 3. The replication products were separated by agarose gel electrophoresis and then excised from the gel for determination of the extent of incorporation of radioactive precursor. The sequence of the wild-type adenovirus terminus is shown at the bottom of the figure, and the specific base substitutions are shown above. For clarity of presentation, the replication activities of the mutants were classified into three groups as follows: ++, indicates replication activity >95% of the wild-type level; +, indicates replication activity 30 to 80% of the wild-type level; −, indicates replication activity <10% of the wild-type level. Underlined segments represent sequences that are highly conserved among adenovirus serotypes.

DISCUSSION

It has been known for several years that the initiation of adenovirus DNA replication on duplex DNA templates requires the presence of specific nucleotide sequences and that the essential sequences must be positioned near the end of the template (10, 19, 26, 38, 46, 47). Analysis of the in vitro replication of templates containing deletion and base substitution mutations resulted in the identification of two sequence domains that are important for origin function (19, 38, 47). One of these domains, the minimal origin, is absolutely required for basal origin activity. The second domain, which contains the recognition site for a cellular protein (NF-I), greatly increases the efficiency of the initiation reaction (19, 38, 47). In the studies reported here, we confirmed and extended these findings by analyzing a large number of deletion and point mutations in the adenovirus origin. Our data indicate that the origin contains an additional accessory sequence domain not identified in previous studies. Thus, the origin consists of at least three functionally distinct domains that we have called A, B, and C. Templates that lack all these domains are totally devoid of replication activity in vitro. Templates containing domain A alone replicate with an efficiency about 3% of that observed with the complete origin. The presence of domain B stimulates replication by more than 10-fold, and the presence of both domains B and C stimulates replication by more than 30-fold. Our data also indicate that domains B and C are unable to support significant initiation of DNA replication in the absence of the minimal origin. For example, single base substitution mutations at nucleotides 17 and 18 in domain A reduce the efficiency of initiation and replication to virtually undetectable levels even though domains B and C are present and unmodified. Finally, the observation that the deletion mutant pU*dl*51 replicates as efficiently as constructs containing several hundred base pairs of the adenovirus

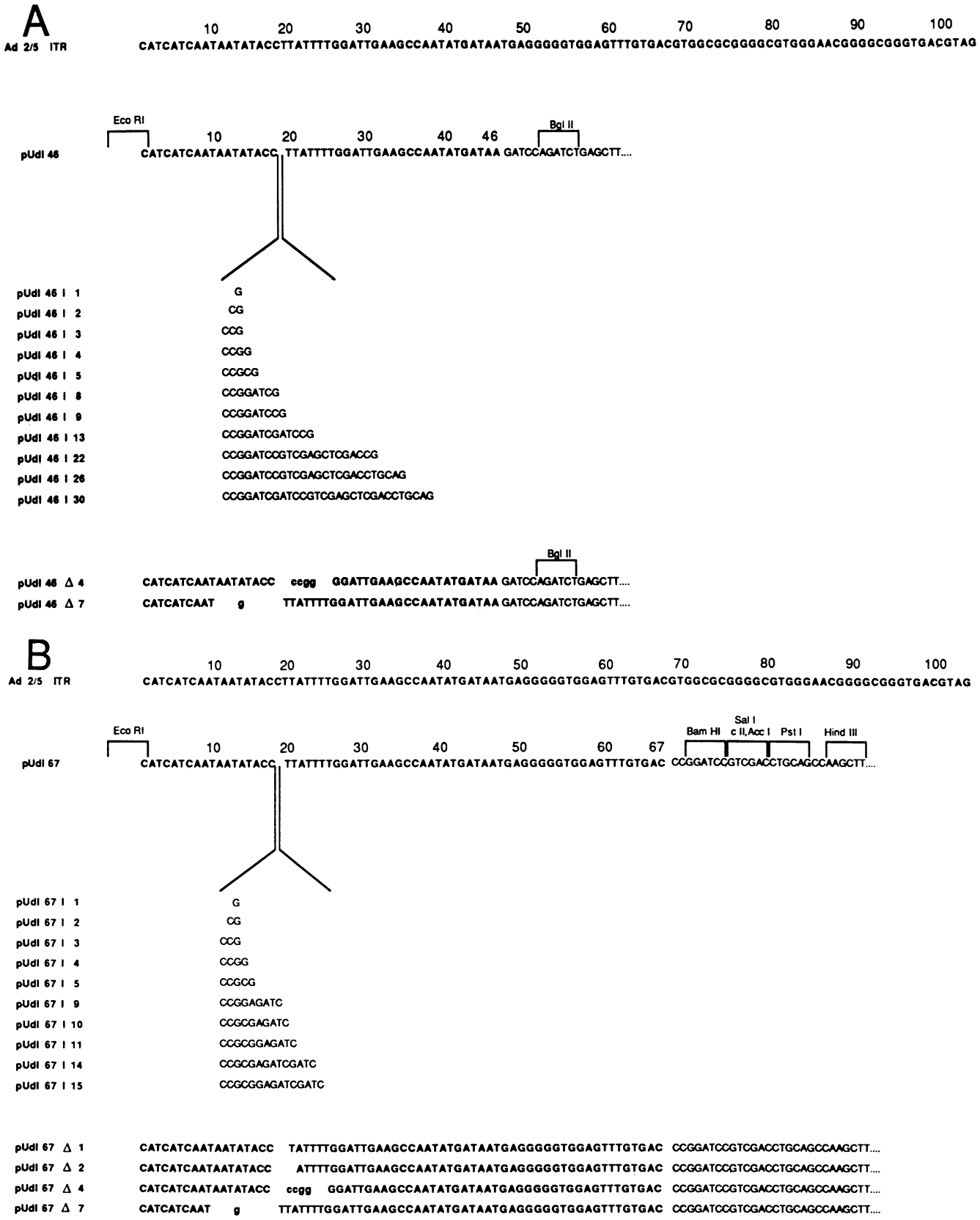

FIG. 8. Sequences of spacing mutants. (A) *dl*46 series of spacing mutants. These mutants contain origin sequence domains A and B but lack a complete domain C. (B) *dl*67 series of spacing mutants. These mutants contain all three origin sequence domains. The viral DNA segments in both series of spacing mutants were cloned into the polylinker site of pUC9. Boldface capital letters indicate adenovirus DNA sequence. Other letters indicate pUC9 DNA sequence or inserted bases. The sequence of the terminus of the adenovirus genome (serotypes 2 and 5) is shown at the top of the figure. ITR, Inverted terminal repetition.

terminal sequence strongly suggests that all the essential sequence domains of the origin lie within the first 51 bp of the viral genome. In vivo studies have also demonstrated the importance of domain B for efficient DNA replication (22). The smaller effect of domain C has not yet been observed in vivo, presumably because of the difficulty in accurately quantitating in vivo replication efficiencies.

The analysis of point mutations, while not completely exhaustive, resulted in the identification of specific nucleotides within each sequence domain that are critical for origin function. We focused most of our attention on domain B to better define the critical nucleotides required for the interaction of NF-I with the origin. The optimal recognition site for NF-I (TTGGCNNNNNGCCAA) appears to consist of two symmetrical half sites separated by a spacer of five nucleotides (13, 18, 23, 38, 40, 41; Rosenfeld et al., in press). The NF-I recognition site in the Ad5 origin (TTG GATTGAAGCCAA) differs from the canonical sequence at only one nucleotide. Base substitution mutations in domain B that significantly reduced the efficiency of initiation were localized within each of the two half sites of the NF-I recognition sequence, confirming the importance of NF-I binding in the initiation of replication. None of the mutations in the spacer region affected initiation efficiency. We also identified two mutations (pUpm20 and pUpm21) in domain B which mapped outside the NF-I consensus recognition sequence but had modest negative effects on initiation of replication in vitro. It is unlikely that these mutations affect the affinity of NF-I for the origin. It is possible that they perturb the binding of some other (undiscovered) initiation protein or that they alter the local structure of the DNA in a manner that reduces the efficiency of some step in the initiation process.

The requirement for specific sequences in domains A and C implies the existence of site-specific DNA-binding proteins that recognize these sequences. It has been reported that the complex of the adenovirus preterminal protein and DNA polymerase binds specifically to a region of domain A (39); however, this result has not been confirmed by other laboratories (1; unpublished data). In the accompanying paper (41), we report the discovery of two cellular proteins that bind with high specificity to sequences within domains A and C.

Our data provide evidence that the distance between the minimal origin and the accessory domains B and C is critical for optimal origin function. The insertion or deletion of only one or two nucleotides between the minimal origin and the accessory sequence block is sufficient to abolish the stimulatory effect of the accessory domains almost completely. These results are in general agreement with data obtained by examination of recombinant adenovirus origins in which the normal NF-I site was replaced with a cloned cellular NF-I-binding site (1). The exquisite sensitivity of the initiation reaction to alterations in the spacing between the minimal origin and accessory domains suggests that there are specific interactions between the proteins that bind to the two regions. The mutants described in this paper should be useful for further investigation of this possibility.

The results reported in this paper, as well as previous studies, have revealed a considerable complexity to the genetic organization of the adenovirus origin of replication. This is probably not surprising in view of the complexity of the initiation reactions that have been observed in the better-studied procaryotic systems (3; M. Dodson, H. Echols, S. Wickner, C. Alfano, K. Mensa-Wilmot, B. Gomes, J. Lebowitz, J. D. Roberts, and R. McMacken, Proc. Natl. Acad. Sci. USA, in press). In these systems the initiation process clearly involves the generation of large nucleoprotein complexes built upon specific protein-DNA and protein-protein interactions. Such interactions serve to determine the specificity of origin selection and to assemble the replication proteins necessary to establish a replication fork. It seems likely that further analysis of the adenovirus system may provide insights into the molecular events involved in initiation of DNA replication in animal cells.

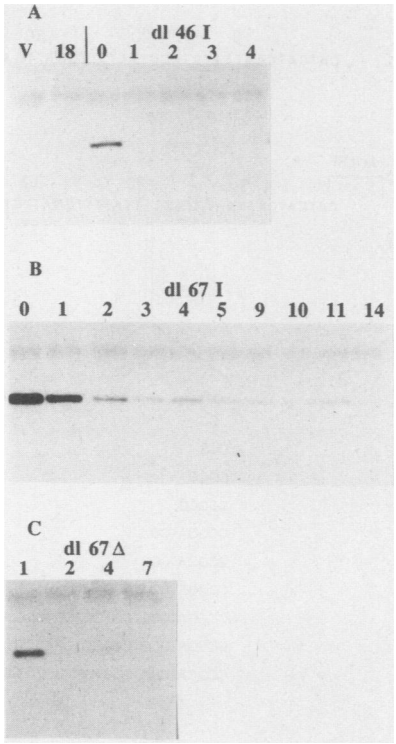

FIG. 9. Initiation of DNA replication with spacing mutants. The mutant templates were tested for their ability to support synthesis of 80-kDa pTP-dCMP complexes in vitro as described in the legend to Fig. 4. (A) Initiation assays with insertion mutants of the *dl*46 series. Lane V, pUC9 as the template; lane 18; pU*dl*18 as the template; lanes 0 to 4, mutants with insertions of 0 to 4 bp. Mutants with insertions larger than 4 bp (5, 8, 9, 13, 22, 26, and 30 bp) showed approximately the same efficiency of initiation as pU*dl*46I4 (data not shown). (B) Initiation assays with insertion mutants of the *dl*67 series. The numbers above each lane represent the size of the insertion mutation. (C) Initiation assays with mutants containing small internal deletions in the neighborhood of nucleotide 19. The numbers above the lanes represent the size of the deletion mutation. See Fig. 8 for the detailed structures of these mutants.

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